

Embryonic and post-embryonic utilization and subcellular localization of the nuclear receptor SpSHR2 in the sea urchin

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SUMMARY

SpSHR2 (*Strongylocentrotus purpuratus* steroid hormone receptor 2) is a nuclear receptor, encoded by a maternal RNA in the sea urchin embryo. These maternal SpSHR2 transcripts, which are present in all cells, persist until the blastula stage and then are rapidly turned over. A small fraction of the embryonic SpSHR2 protein is maternal, but the majority of this nuclear receptor in the embryo is the product of new synthesis, presumably from the maternal RNA after fertilization. In agreement with the mRNA distribution, the SpSHR2 protein is also detected in all embryonic cells. Contrary to the RNA though, the SpSHR2 protein persists throughout embryonic development to the pluteus stage, long after the mRNA is depleted. Following fertilization and as soon as the 2-cell stage, the cytoplasmic SpSHR2 protein enters rapidly into the embryonic nuclei where it appears in the form of speckles. During subsequent stages (from fourth cleavage onward), SpSHR2 resides in speckled form in both the nucleus and the cytoplasm of the embryonic cells. The cytoplasmic localization of SpSHR2 differs between polarized and non-polarized cells, maintaining an apical position in the ectoderm and endoderm versus a uniform

distribution in mesenchyme cells. Following the end of embryonic development (pluteus stage), the SpSHR2 protein is depleted from all tissues. During the ensuing four weeks of larval development, the SpSHR2 is not detected in either the larval or the rudiment cells which will give rise to the adult. Just prior to metamorphosis, at about 35 days post-fertilization, the protein is detected again but in contrast to the uniform distribution in the early embryo, the larval SpSHR2 is specifically expressed in cells of the mouth epithelium and the epaulettes. In adult ovaries and testes, SpSHR2 is specifically detected in the myoepithelial cells surrounding the ovarioles and the testicular acini. Nuclear SpSHR2 in blastula extracts binds to the C1R hormone response element in the upstream promoter region of the *CyIIIb* actin gene indicating that the latter may be a target of this nuclear receptor in the sea urchin embryo.

Key words: Sea urchin embryonic development, SpSHR2 orphan nuclear receptor, Maternal RNA, Subcellular protein localization, Tissue specific larval expression

INTRODUCTION

Members of the steroid receptor superfamily along with several orphan receptors have been isolated from a variety of animals (Laudet, 1997). These potent transcriptional regulators modulate responses to external stimuli and complex processes including cell fate specification and pattern formation. Furthermore, a number of orphans have been shown to function specifically during early embryogenesis, whereas others are required in multiple stages of development (Thummel, 1995).

Two genes that belong to the nuclear receptor superfamily have been identified in the sea urchin so far. These genes, both encoding orphan members of the family, are the SpCOUP-TF (Chan et al., 1992) which is highly conserved among different species and the SpSHR2 (Kontrogianni-Konstantopoulos et al., 1996). These transcription factors

bind specifically to a regulatory *cis*-acting element, called C1R (Niemeyer and Flytzanis, 1993), present in the upstream promoter region of the *CyIIIb* actin gene. The *CyIIIb* actin (Flytzanis et al., 1989) is exclusively expressed in the cells of the embryonic aboral ectoderm (Cox et al., 1986), reaching peak RNA levels at the pluteus stage. The hormone response element C1R is involved in the spatial repression of *CyIIIb* in cell types other than the aboral ectoderm. In the aboral ectoderm of the late embryo, in co-operation with the upstream element E1, C1R seems to act as a positive regulatory site (Xu et al., 1996).

SpSHR2 is homologous to the human testis receptor 2-11 (hTR2-11; Chang and Kokontis, 1988; Chang et al., 1989) and to a lesser extent to the human RXRa (Mangelsdorf et al., 1990). SpSHR2 and TR2-11 share a 79% amino acid identity within their DNA binding domains (DBD) and a 62% identity within their ligand binding domains (LBD). Their overall

amino acid identity is about 50% and thus, we do not classify SpSHR2 as the sea urchin TR2 orthologue, but as a novel member of the steroid-thyroid-retinoic acid receptor superfamily.

The SpSHR2 message is present in the pool of maternal mRNAs, since it is found deposited in the unfertilized egg. It persists up to the blastula stage, sharply declining thereafter (Kontrogianni-Konstantopoulos et al., 1996). This finding is unusual for maternal sea urchin transcripts, which are usually replaced by zygotic counterparts at the blastula stage, as it is the case for many prevalent messages including the tubulin mRNA, the α -histone mRNA and many others (Flytzanis et al., 1982; Davidson, 1986).

Early development proceeds mostly at the expense of maternal proteins and messages which are utilized for translation immediately following fertilization or within a few hours. The translation products of the maternal transcripts are required during early embryogenesis to sustain cell division, growth and differentiation of cells to specific lineages (Kingsley et al., 1993). As most maternal RNAs, SpSHR2 is also translated during the early cleavage stages as shown by in vivo labeling followed by immunoprecipitation (Kontrogianni-Konstantopoulos et al., 1996). SpSHR2 transcripts are present in different isoforms. Characterization of two cDNA clones (4.1.1 and 4.15.13) demonstrated that at least two different SpSHR2 messages are generated, as a result of alternative splicing of the primary transcripts from the single SpSHR2 gene. One of the clones (4.1.1) encodes a protein with the typical structure of a nuclear hormone receptor (Forman and Samuels, 1990; Mangelsdorf et al., 1995), whereas the other clone (4.15.13) encodes a truncated isoform, which is missing the putative ligand binding domain of the receptor.

In this study we analyzed the distribution of the SpSHR2 mRNA and protein during embryogenesis and investigated the subcellular localization of the receptor in the developing embryo and its specific expression in the larva and adult animal. With the use of specific antibodies and confocal microscopy we were able to observe the intracellular movements of this transcription factor from the egg cytoplasm to the early embryonic nuclei, a result of an apparent activation following fertilization. We also determined that the endogenous SpSHR2 receptor, present in blastula nuclear extracts, binds specifically to the C1R hormone response element of the CyIIIb actin gene.

MATERIALS AND METHODS

Embryonic cultures

Sea urchins were purchased from Marinus, Inc. and Pacific Biomarine, Inc. (Long Beach, CA). The animals were kept at 12°C in an aquarium and fed kelp (*Macrocystis pyrifera*). Gametes were obtained by intracoelomic injection of 0.5 M KCl. The embryos were cultured at 15°C with constant aeration in filtered sea water. Larvae were fed with *Rhodomonas* sp. and cultured until metamorphosis as previously described (Flytzanis et al., 1985; Leahy, 1986).

Whole mount in situ hybridization

Whole mount in situ hybridization was carried out according to the method of Harkey and Whitely (1983), with the modifications described by Vlahou et al. (1996). As probes, either the 3' untranslated region (UTR) or the ligand binding domain (LBD) of the SpSHR2

clone 4.1.1 were utilized. Both fragments were subcloned in the PCRTMII plasmid (Invitrogen). To prepare the UTR sense and antisense SpSHR2 probes, the UTR-carrying plasmid was linearized with the restriction enzymes *KpnI* and *XmnI* and transcribed by Sp6 and T7 polymerases, respectively.

Production of polyclonal antibodies

A PCR amplified LBD fragment of clone 4.1.1 corresponding to nucleotides 1,178-2,120 (encoding 314 amino acids), was fused downstream of the malE gene in the pMAL-c2 vector which encodes the *Escherichia coli* maltose-binding protein (MBP; New England Biolabs). The produced fusion protein had a molecular mass of 85 kDa and it was affinity purified by an amylose resin column. The purified protein was used to immunize two rabbits after mixing with Freund's complete adjuvant. The rabbits were injected intradermally with 0.3 mg of the antigen each time. After the first injection three boosters followed, each with a fifteen-day interval. A blood sample was collected each time and the obtained serum was tested by protein blot (western) analysis.

In vitro transcription-translation

The 4.1.1 cDNA clone was linearized with the restriction enzyme *KpnI* and the linear plasmid was used as template for in vitro transcription using the T3 RNA polymerase (Ambion, T3 Message MachineTM kit). The produced RNA, as well as a control RNA encoding luciferase, were then utilized as templates for in vitro translation, using rabbit reticulocyte extracts and the kit provided by Promega.

Immunoprecipitation

Staphylococcus aureus cells were washed using TSNDT (20 mM Tris-HCl, pH 7.4, 0.1% SDS, 1% NaCl, 1% deoxycholate, 1% Triton X-100) by repeated cycles of pelleting and resuspending at 4°C. 1 μ l of the SpSHR2 antibody or the preimmune serum was incubated with the in vitro translation product of clone 4.1.1 or the luciferase protein in TSNDT at a final volume of 100 μ l, for 2 hours, at 4°C. At the end of the incubation period, 10 μ l of the washed *S. aureus* cells were added and incubation for another hour at 4°C followed. The formed complexes were then pelleted through a sucrose cushion (1 M sucrose, 20 mM EDTA, 1% deoxycholate, 1% Triton X-100, 1% NaCl, 20 mM Tris-HCl, pH 7.4) twice. The pellets were dissolved in 20 μ l SDS-sample buffer and analyzed in a 10% acrylamide gel. The dried gel was exposed to X-ray film for 10 hours.

Preparation of embryonic protein extracts and protein blot analysis

Protein extracts from unfertilized eggs, embryos and isolated adult organs were prepared as follows: after several washes with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free sea water they were resuspended in an equal volume of 2 \times SDS-sample buffer and subsequently homogenized. Any insoluble material was removed by centrifugation at 10,000 *g* and the supernatants were boiled for 5 minutes. The protein lysates were then separated in a 10% SDS gel or stored at -80°C. Protein extracts corresponding to either 10,000 eggs or embryos were loaded per lane, whereas no quantitation was attempted for the protein lysates of the adult tissues. The separated proteins were then transferred onto nitrocellulose sheets and incubated with either the SpSHR2 antibody or the preimmune serum at a dilution of 1:1,500 for 4 hours at RT. An alkaline phosphatase-conjugated goat anti-rabbit antibody (Bio-Rad, Immune Blot Assay kit) was used as the secondary antibody. The immune complexes were visualized by using the chromogenic substrates NBT and BCIP (Bio-Rad).

Indirect immunofluorescence of whole mount embryos and larval sections

Embryos at different developmental stages were collected, washed twice with $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free sea water, suspended in 100% methanol

and incubated for 1 hour on ice. Following the fixation period the embryos were resuspended in PBS (10 mM PB, pH 7.2, 150 mM NaCl) and stored at 4°C. Larvae at different developmental stages were fixed in Bouin's fixative for 24 hours, resuspended in 70% ethanol, embedded in paraffin and sectioned (5 µm thick sections). After deparaffinization and rehydration they were washed with PBS. Blockage of the free reactive groups was achieved by incubation in PBS plus 0.1% Tween-20, 4% goat serum and 2% BSA, for 4 hours at RT. Primary antibodies were added at the following dilutions: SpSHR2 1:1,000; preimmune serum 1:1,000 and chicken anti-lamin 1:100 in PBS plus 0.05% Tween-20, 2% goat serum and 2% BSA and incubated for 12 hours at 4°C. Several washes with PBST plus 0.05% Tween-20 were performed and incubation with the secondary antibodies, anti-rabbit Texas Red 1:100 (Pierce) and anti-chicken FITC (KPL) 1:100, followed in the same solution, for 1 hour at RT in the dark. After the end of the incubation, the embryos or the larval sections were washed with PBST plus 0.1% Tween-20 and mounted in 0.1% phenylenediamine in 1:9 PBS:glycerol. For the competition experiment the SpSHR2 antibody was preincubated with 10 µg of the LBD antigen in a total volume of 100 µl of PBS for 4 hours at RT. The embryos were then observed using a laser scanning confocal microscope (Summers et al., 1993) and the larval sections using an Axiophot fluorescent microscope. All embryonic and larval stages were treated under the same conditions, both in the immunofluorescence and microscopic analyses, and thus the observed staining differences should reflect changes in the amount of endogenous protein.

Immunocytochemistry

Ovaries and testes were isolated from adult sea urchins, fixed in Bouin's solution, paraffinized and sectioned. After deparaffinization and rehydration the sections were processed for immunocytochemistry utilizing the Vectastain Elite ABC KIT and the DAB Substrate Kit for Peroxidase (Vector). The SpSHR2 antibody was used at a dilution of 1:2,000 and the myosin antibody was used at a dilution of 1:750. After color development, the samples were mounted with Glycergel (DACO) for microscopic observation.

Electrophoretic mobility shift assay (EMSA)

Double stranded oligonucleotide carrying the C1R HRE 5'cattTGACCCgcTGACCTttgcta (Chan et al., 1992), was 5' end-labeled using T4 polynucleotide kinase and [γ - 32 P]ATP. Blastula nuclear extracts were prepared as described by Niemeyer and Flytzanis (1993). Each binding reaction (20 µl) contained 10 µl probe mix (20,000 cpm labeled C1R probe, 0.25 µg/ml dI:dC, 0.25 µg/ml dA:dT, 20 mM Hepes, pH 7.6, 0.5 mM DTT, 75 mM KCl, 5 mM MgCl₂), 1 µl blastula nuclear extract, and either a 500-fold excess of C1R cold competitor (2.5 ng), or a 500-fold excess of an unrelated oligonucleotide as competitor (2.5 ng), and 1 µl of the SpSHR2 antibody or the preimmune serum. The binding reactions were

incubated at RT for 30 minutes, except the blocking reaction for which the extracts were preincubated with the SpSHR2 antibody for 10 minutes on ice. Following the incubation period, 2 µl of loading buffer (15% Ficoll, 0.25% Bromophenol Blue, 0.25% Xylene Blue) were added and the reactions were analyzed in an 8% acrylamide gel. Dried gels were exposed to X-ray film for about 10 hours.

RESULTS

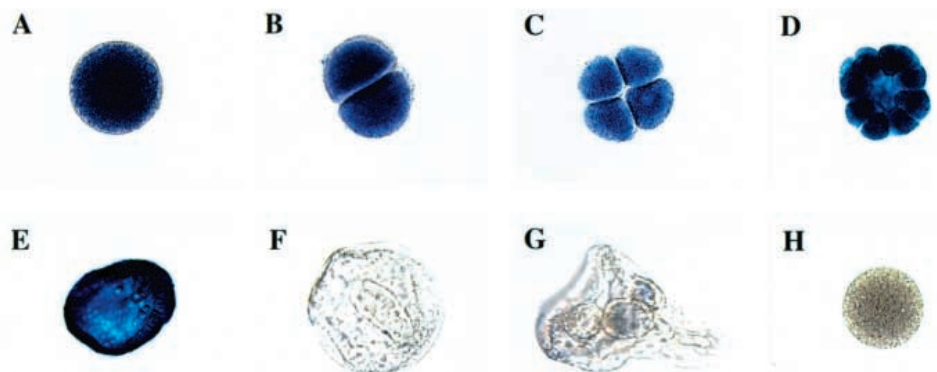
The maternal SpSHR2 RNA is uniformly distributed in the early embryo

RNA-blot and reverse-transcription PCR experiments have shown that the SpSHR2 transcripts are maternal, detected in the total RNA of unfertilized eggs and persist up to the blastula stage (Kontogianni-Konstantopoulos et al., 1996). To study the distribution of the maternal SpSHR2 mRNA within the embryo, whole mount in situ hybridization was employed. Sense and antisense riboprobes were synthesized, which correspond to the common 3' UTR of the SpSHR2 transcripts and thus detect all splice variants (Fig. 1). These experiments show that the SpSHR2 mRNA is evenly distributed in the unfertilized egg and detected in all cells of the developing embryo until the blastula stage (Fig. 1A-E), disappearing shortly thereafter (Fig. 1F,G). Moreover, when embryos of intermediate developmental stages were analyzed (36-hour early gastrulae), a homogeneous disappearance of the SpSHR2 message was observed in all different embryonic cell types (not shown).

Production of SpSHR2 antibodies directed against the LBD of the protein

To examine the expression pattern and localization of the SpSHR2 receptor in the developing sea urchin embryo and larva, polyclonal antibodies against a major part of the receptor's LBD (314 amino acids) were raised (Fig. 2A). The produced antibodies detect only the isoform encoded by the 4.1.1 clone, since the other splice variant (4.15.13) does not contain this fragment of the LBD. Their specificity was tested by an immunoprecipitation assay. The SpSHR2 antibodies specifically recognize the in vitro translation product of the SpSHR2 mRNA transcribed from clone 4.1.1 (Fig. 2B, lane 2, arrowhead), whereas the preimmune serum does not (Fig. 2B, lane 3). The faster migrating bands that are also immunoprecipitated by the SpSHR2 antibody may be the result of shorter in vitro synthesized RNAs, unfinished polypeptide

Fig. 1. Distribution of the SpSHR2 maternal message in the *S. purpuratus* embryo. Whole mount in situ hybridization was performed, utilizing a digoxigenin-UTP labeled anti-sense (A-G) or sense (H) probe that corresponds to the 3' UTR of the SpSHR2 gene. (A) Unfertilized egg, (B) 2-cell stage, (C) 4-cell stage, (D) 16-cell stage, (E) blastula, (F) gastrula, (G) pluteus, (H) unfertilized egg. The images were photographed under $\times 40$ magnification.



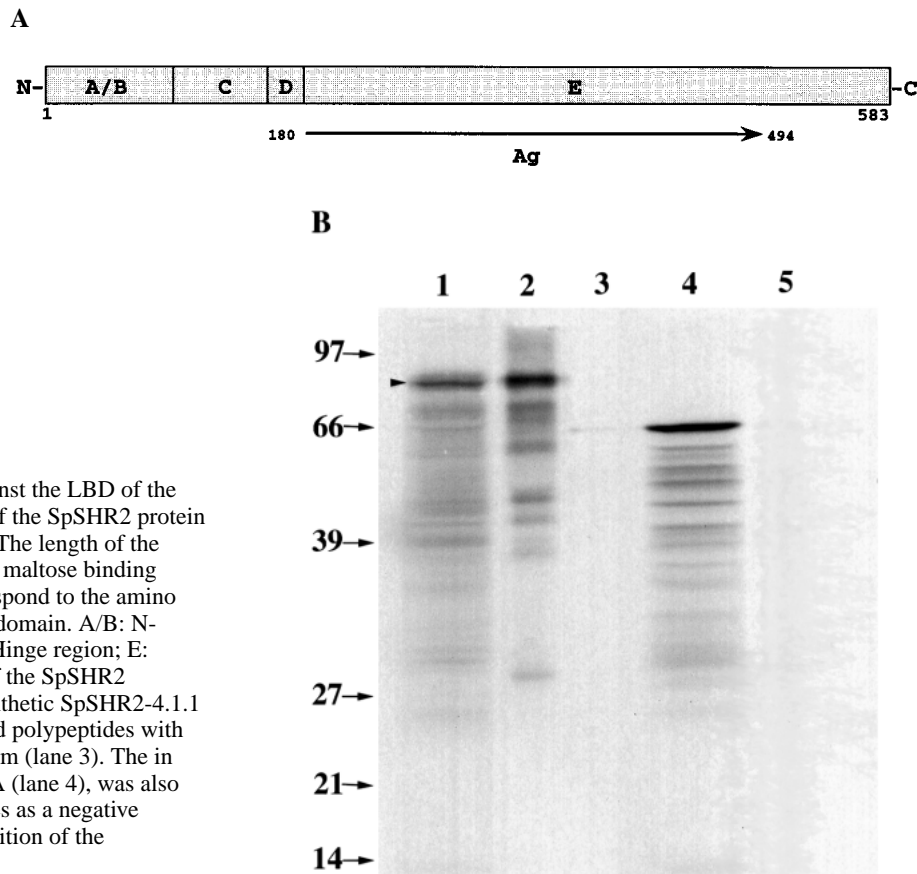


Fig. 2. Generation of polyclonal antibodies against the LBD of the SpSHR2 receptor. (A) Schematic presentation of the SpSHR2 protein showing the structural domains of the receptor. The length of the arrow spans the portion of the LBD fused to the maltose binding protein and used as antigen. The numbers correspond to the amino acid positions at the beginning and end of each domain. A/B: N-terminal domain; C: DNA binding domain; D: Hinge region; E: Ligand binding domain. (B) Specificity assay of the SpSHR2 antibodies. SDS-PAGE of in vitro translated synthetic SpSHR2-4.1.1 mRNA (lane 1, arrowhead). Immunoprecipitated polypeptides with SpSHR2 antibodies (lane 2), or preimmune serum (lane 3). The in vitro translation product of the luciferase mRNA (lane 4), was also immunoprecipitated with the SpSHR2 antibodies as a negative control (lane 5). Arrows mark the migration position of the molecular mass standards.

chains or degradation products. The in vitro translation product of the luciferase mRNA (Fig. 2B, lane 4) was used as a control and as expected it is not precipitated by the SpSHR2 antibodies (Fig. 2B, lane 5).

SpSHR2 is stored in the egg as a maternal protein

Protein blots using the SpSHR2 antibodies and equivalent amounts of protein extracts from unfertilized eggs, blastulae, gastrulae and plutei, revealed the presence of the protein in all embryonic stages. The SpSHR2 receptor migrates in the gels with an apparent molecular mass of around 70 kDa, whereas the open reading frame (ORF) of clone 4.1.1 predicts a protein with a molecular mass of around 65 kDa. This size difference between the apparent (as indicated by SDS-PAGE) and the predicted (as estimated by the ORF) molecular masses is not uncommon and may be due to some post-translational modification that the SpSHR2 protein undergoes in vivo. Receptor molecules are detected in the unfertilized egg and thus SpSHR2 is a maternal protein (Fig. 3, lane E). Furthermore, it is present throughout embryonic development, up to the pluteus stage (Fig. 3, lanes B, G and P). The maternal protein in the unfertilized egg is found in seemingly lower amount compared to the embryonic stages (blastula to pluteus). The additional SpSHR2 protein of the embryonic stages should arise from translation of the stored maternal mRNA. It is also conceivable that additional SpSHR2 transcripts accumulate in early embryos, by zygotic gene expression. The SpSHR2 receptor persists up to the pluteus stage, long after the mRNA

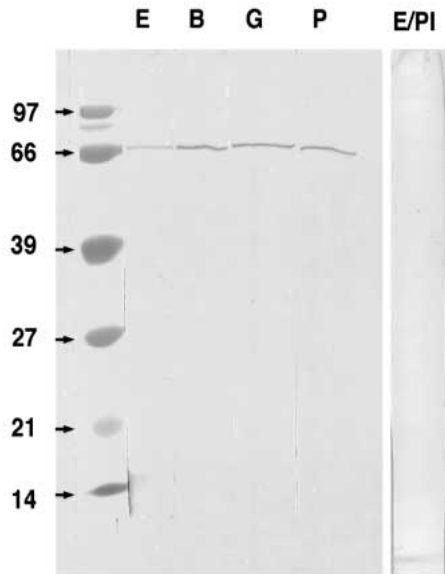


Fig. 3. Maternal and embryonic SpSHR2. Western blot utilizing equivalent amounts of protein extracts from different developmental stages of *S. purpuratus* embryos. The SpSHR2 antibody was used as primary and an anti-rabbit alkaline-phosphatase conjugated antibody was used as secondary. E: unfertilized egg, B: blastula, G: gastrula, P: pluteus, PI: preimmune serum. Arrows mark the migration position of the molecular mass standards.

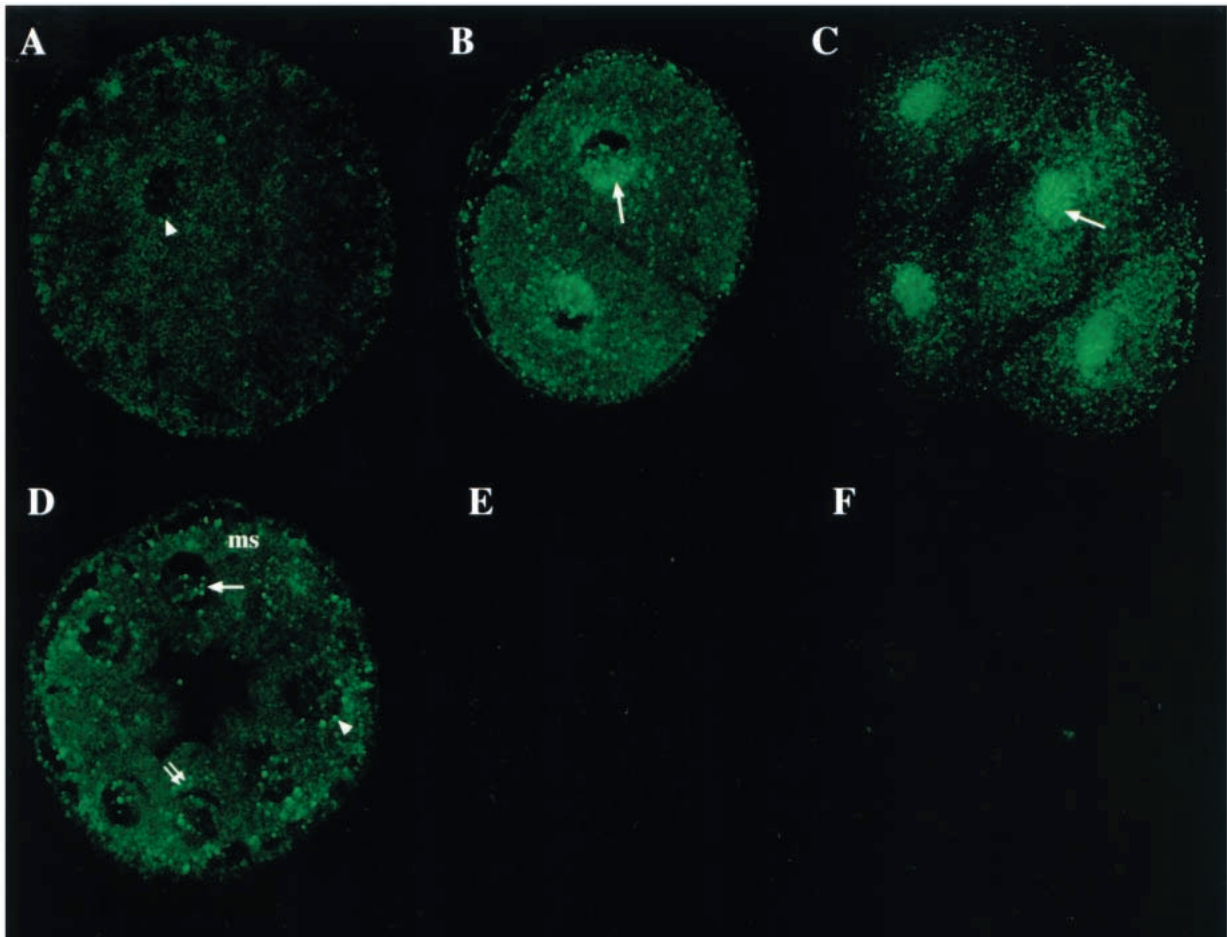


Fig. 4. Analysis of the localization pattern of the SpSHR2 receptor in the early embryo utilizing immunofluorescence combined with confocal microscopy. As primary antibody either the SpSHR2 antibody (A-D) or the preimmune serum (E) was used. An anti-rabbit FITC antibody was utilized as secondary. As negative control preincubation of the SpSHR2 antibody with the purified recombinant antigen (LBD) was performed (F). (A) unfertilized egg, (B) 2-cell stage, (C) 4-cell stage, (D-F) 16-cell stage. ms: mesomeres. Single arrows point to nuclear, whereas the arrowheads and double arrow point to perinuclear and cytoplasmic SpSHR2 foci respectively. All images were analyzed at $\times 40$ magnification. The micrographs shown are single $0.5\ \mu\text{m}$ optical sections from a series of optical sections.

is depleted, indicating a lasting function throughout embryogenesis. As a control experiment for the western blots, the SpSHR2 antibody was preincubated with the purified recombinant antigen (LBD) and then applied to blots of total embryonic protein extracts. In this case the single band was not observed (data not shown), indicating that the 70 kDa protein corresponds to SpSHR2.

Subcellular localization of SpSHR2 in early embryos

The localization of the SpSHR2 receptor in the early sea urchin embryonic cells was investigated by immunofluorescence using confocal microscopy (Fig. 4). There is hardly any detectable amount of protein in large oocytes (not shown), whereas in the unfertilized mature eggs SpSHR2 molecules were detected in small amounts in the cytoplasm and concentrated around the pronucleus (Fig. 4A, arrowhead). In the 2- and 4-cell stage embryos, the maternal and presumably the newly synthesized SpSHR2 receptor molecules accumulate into the embryonic nuclei. As previously shown by immunoprecipitation of *in vivo* labeled proteins (Kontogianni-

Konstantopoulos et al., 1996) newly synthesized SpSHR2 accumulates at these stages. Thus, following fertilization, a large amount of the protein either enters the nucleus or is found concentrated in the perinuclear region and only a small amount remains scattered in the cytoplasm (Fig. 4B-C, arrows). As the embryo proceeds to the later cleavage stages, we do not observe this high nuclear concentration of SpSHR2. In 16-cell stage embryos, the protein is both nuclear (Fig. 4D, arrow and arrowhead) and cytoplasmic (Fig. 4D, double arrow) and seems to be equally distributed in all cells along the embryonic animal/vegetal axis. The preimmune serum, used as a control, does not produce any detectable signal (Fig. 4E). Furthermore, in a competition experiment where the SpSHR2 antibody was preincubated with the purified recombinant SpSHR2 antigen no signal was obtained (Fig. 4F).

At the blastula stage (Fig. 5A), the receptor molecules are seen in foci either within the nucleus (Fig. 5D, arrow), on the nuclear lamina (Fig. 5D, double arrow) or in the cytoplasm (Fig. 5D, arrowhead), as shown by double labeling with the SpSHR2 antibody and a lamin specific antibody (Holy et al.,



Fig. 5. Spatial distribution of the SpSHR2 receptor in the later embryonic stages of *S. purpuratus* embryos. Double labeling of embryos was performed using the SpSHR2 and a lamin-B antibody. As secondary antibodies, anti-rabbit Texas Red and anti-chicken FITC were used, respectively. The images were analyzed in the confocal microscope, at $\times 40$ magnification and represent single $0.5\ \mu\text{m}$ optical sections from a series of sections. (A) Blastula, (B) gastrula, (C) pluteus, (D) magnification of blastula primary mesenchyme cells, (E) magnification of gastrula epithelial cells, (F) magnification of pluteus aboral ectoderm cells, (G) magnification of the pluteus mouth. Single arrows point to nuclear, whereas the arrowheads and double arrow point to apically localized cytoplasmic and perinuclear SpSHR2 foci, respectively.

1995), which marks the periphery of the nucleus. That the localization of the receptor molecules was intracellular, was confirmed by consecutive $0.5\ \mu\text{m}$ optical sections through the embryo, in which the receptor foci present in the nucleus, on the nuclear lamina or in the cytoplasm were always observed in these respective cellular compartments of the cells present in each section. Furthermore, the SpSHR2 foci observed in the middle of the blastocoel (Fig. 5A) belong to ingressed primary mesenchyme cells, whose nuclei are out of focus on this optical section (and thus not labeled by the lamin antibody) and are not associated with the extracellular matrix of the blastocoel.

At the gastrula stage (Fig. 5B) SpSHR2 foci are detected in

both the nucleus and the cytoplasm of the embryonic cells as well (Fig. 5E, arrow and arrowhead, respectively). Closer examination of the intracellular distribution of the receptor in the primary mesenchyme cells of the blastula (Fig. 5D, arrowhead) and the polarized ectodermal cells of the blastula and gastrula (Fig. 5E, arrowhead), revealed that in the latter the cytoplasmic fraction of the receptor is concentrated in the apical side of the cells. In the ingressed primary mesenchyme cells though, no such polarized cytoplasmic localization was observed. At the pluteus stage (Fig. 5C) the amount of both nuclear and cytoplasmic SpSHR2 is seemingly lower per individual cell (Fig. 5F, arrows and arrowhead, respectively)

compared to earlier stages, in all cell types except the cells of the oral ectoderm that form the pluteus mouth (Fig. 5G, arrowhead). These polarized epithelial cells still exhibit the profound apical accumulation of the receptor.

It is worth mentioning that the immunofluorescence experiments with all embryonic stages were performed under the same conditions i.e. type of fixation, primary antibody or preimmune serum dilution, secondary antibody dilution, incubation periods, microscope voltage settings, etc. Thus, the results obtained from different developmental stages are comparable.

Cell-type specific expression of SpSHR2 in late larvae prior to metamorphosis

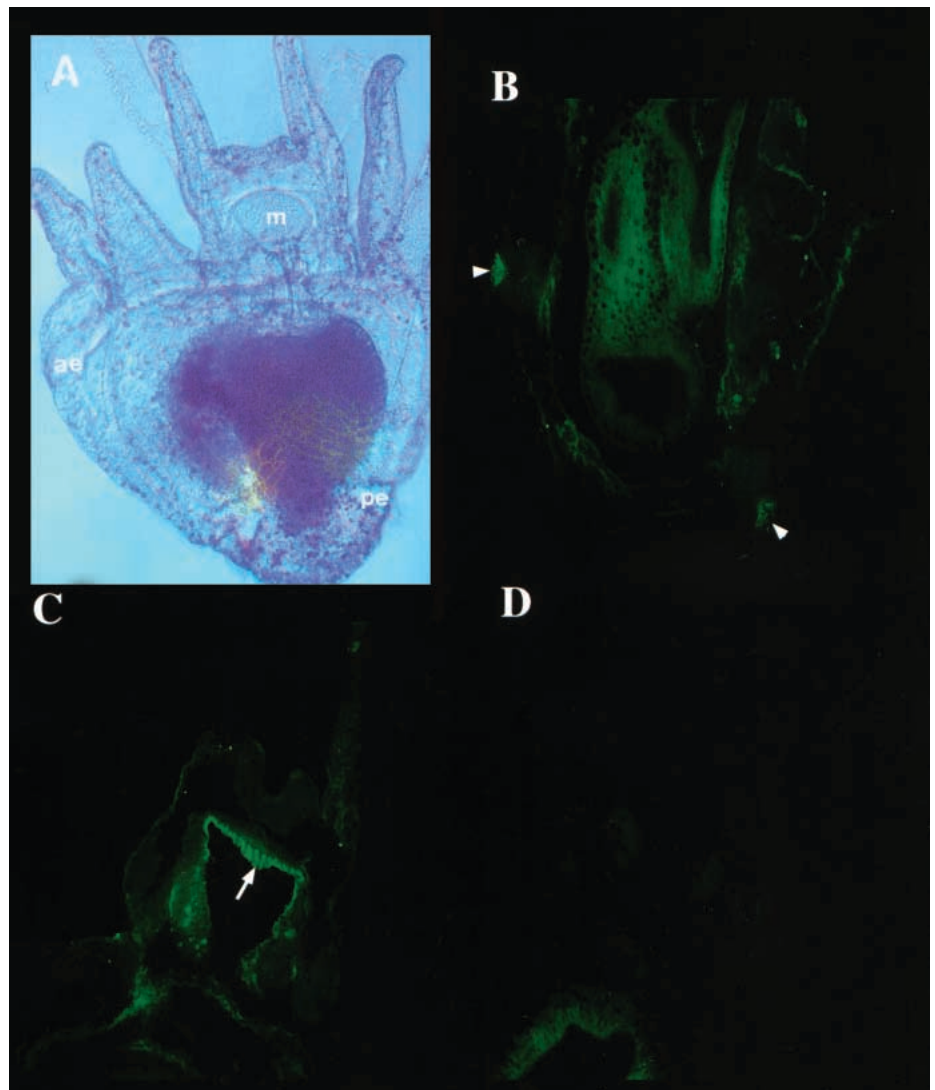
To examine whether the SpSHR2 gene is activated in post embryonic sea urchin tissues, immunofluorescence microscopy was performed with larval sections from different developmental stages (Fig. 6). No positive signal was obtained with one, two, three and four week old larvae consistent with the late embryonic depletion of the protein (not shown). However, at thirty five days post fertilization SpSHR2 protein is specifically detected in the epaulettes (Fig. 6B, arrowheads)

and the mouth epithelium (Fig. 6C, arrow) of the swimming larva. When preimmune serum was used, instead of the SpSHR2 antibodies, no signal was obtained from the epaulettes or the mouth (Fig. 6D). The larval gut emits background fluorescence at these stages as seen with either the SpSHR2 antibody (Fig. 6B), or the preimmune serum (Fig. 6D). Since the larval gut background is detected even in the absence of any primary antibodies (not shown), we consider the gut staining to be an artifact produced by the secondary antibody.

SpSHR2 is expressed by specific cell types in adult tissues

Expression of the SpSHR2 receptor in adult sea urchins was studied with protein blots, using extracts from several organs. The SpSHR2 receptor was detected in all the different organs examined, i.e. lantern muscle, tubefeet, intestine, coelomocytes, ovaries and testes (Fig. 7). To investigate further the cell type which expresses SpSHR2 in the adult tissues, sections from ovaries and testes were utilized in immunocytochemistry experiments. The ovaries were obtained after spawning of the eggs and thus only a small number of

Fig. 6. Expression of SpSHR2 receptor in the developing *S. purpuratus* larva. (A) Light microscope image of a 35-day-old *S. purpuratus* larva; the entire larval body from the tip of the anterior arms to the posterior spans about 500 μm ; m: mouth; ae: anterior epaulettes; pe: posterior epaulettes. Immunofluorescence using SpSHR2 antibody (B-C), or preimmune serum (D) as primary and anti-rabbit FITC antibody as secondary. (B and C) 35-day-old larvae; the arrowheads in B point to the SpSHR2 expressing anterior and posterior epaulettes and the arrow in C points to the SpSHR2 expressing mouth epithelial cells. Staining in the interior of the larva (gut tissue) is an artifact of the secondary antibody (B and D). All larvae are viewed with the anterior towards the top of the picture. The fluorescent images were observed at $\times 20$ magnification.



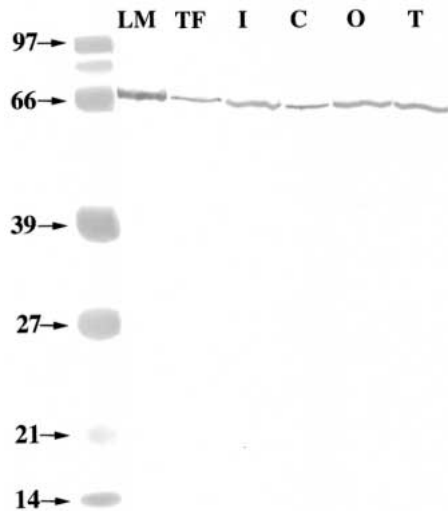


Fig. 7. Expression of SpSHR2 in adult sea urchin tissues. Western blot using SpSHR2 antibodies and tissue extracts from: LM: lantern muscle; TF: tube feet; I: intestine; C: coelomocytes; O: ovaries; T: testes. An anti-rabbit alkaline-phosphatase conjugated antibody was used as secondary. Arrows mark the migration position of the molecular mass standards.

mature ova was present. In sections of ovaries and testes the SpSHR2 receptor was specifically expressed in the smooth muscle epithelial layer of cells which surround the ovarioles and acini, respectively (Fig. 8A,B, arrow). The nature of this cell type was revealed by the specific detection of myosin in the same cells as shown by staining with a myosin antibody (Fig. 8C, arrow). No positive signal was obtained when preimmune serum was used (Fig. 8D).

The SpSHR2 receptor binds to the C1R hormone response element in the upstream promoter of the *CyIIIb* actin gene

EMSA, using nuclear protein extracts from blastula stage embryos and the C1R hormone response element (HRE), which was identified in the upstream regulatory region of the *CyIIIb* actin gene, demonstrated that the SpSHR2 receptor specifically binds to this element (Fig. 9). Two specific C1R-protein complexes were formed (Fig. 9, lane 2, arrowhead) that were competed by excess of unlabeled C1R probe (Fig. 9, lane 3), but not by an unrelated oligonucleotide (Fig. 9, lane 4). Both of these C1R-protein complexes are specifically supershifted by the SpSHR2 antibody (Fig. 9, lanes 5 and 7, arrow), but not by the preimmune serum (Fig. 9, lane 6).

DISCUSSION

The present study deals with the expression, embryonic distribution and subcellular localization of the SpSHR2 nuclear receptor in the developing sea urchin embryo, larva and adult animal. The SpSHR2 mRNA belongs to the pool of maternal messages, since it is found deposited in the unfertilized egg, and persists up to the blastula stage, sharply declining to undetectable levels thereafter. Zygotic transcripts, replacing maternal ones, do not typically accumulate until blastula, which

is the stage that the SpSHR2 mRNA disappears (Flytzanis et al., 1982; Wilt, 1987; Davidson, 1989). Thus, it is conceivable that the SpSHR2 message belongs to a group of maternal transcripts, that are not replenished by zygotic transcription. However, we cannot exclude the possibility that the SpSHR2 gene is activated during early embryonic development, resulting in the synthesis of zygotic RNA and is subsequently turned off before the gastrula stage. It is also possible that there are zygotic transcripts present in the later stage embryos, but at levels below our detection limit and/or extremely unstable.

Similarly to the SpSHR2 message, the protein is synthesized during oogenesis and stored in the cytoplasm of the unfertilized egg. Contrary to the mRNA however, the SpSHR2 protein is detected throughout embryonic development, long after the 24-hour blastula when the mRNA begins to be depleted. This evidence indicates that the SpSHR2 protein is very stable and presumably has a function that persists throughout embryogenesis.

The maternal SpSHR2 protein is present in smaller amounts in the unfertilized egg compared to the blastula stage, found throughout the cytoplasm, seemingly concentrated at the perinuclear region, but not inside the egg's pronucleus. It is presumed to be inactive at this stage. Following fertilization, maternal and presumably the newly synthesized SpSHR2 protein enters into the embryonic nuclei and by the 2- and 4-cell stage, most of it is concentrated in distinct foci either in the nucleoplasm or on the nuclear lamina. At this early stage, there is also detectable SpSHR2 protein in foci outside the nucleus, at the perinuclear region, and in the cytoplasm. The swift translocation and accumulation of the protein into the embryonic nuclei following fertilization, is indicative of a very early nuclear function. Two cell divisions later however, by 16-cell stage, SpSHR2 is no longer preferentially found in the nucleus, as a large amount of it resides also in the cytoplasm. From the 16-cell stage onward, and until SpSHR2 becomes undetectable, it resides in both the nucleus and the cytoplasm. Thus, if there is an anchoring cytoplasmic mechanism for the embryonic SpSHR2 protein, it may not be in place until the embryo reaches the 16-cell stage.

The localization pattern of the receptor resembles the speckled appearance (Spector, 1993) of factors involved in the pre-mRNA splicing. Two other nuclear receptors, the glucocorticoid receptor (GR) (Martins et al., 1991; Yang and DeFranco, 1994) and the hepatocyte nuclear factor 4 (HNF4; Ktistaki et al., 1995) were also shown to localize in specific subnuclear compartments. Furthermore, HNF4 was found to distribute in these domains after activation through tyrosine phosphorylation. Several investigators have tried to elucidate the contents of such intranuclear speckles and have demonstrated that snRNAs, snRNPs, essential splicing factors such as SC-35 and a number of non-snRNP splicing factors are present (Huang and Spector, 1992; Zhang et al., 1994; Hedley et al., 1995). Furthermore, electron microscopic analysis has revealed that such speckles are organized in a latticework that corresponds either to active sites of transcription or assembly and/or storage sites of splicing factors (Jackson et al., 1993; Leonhardt and Cardoso, 1995; Huang and Spector, 1996). In accordance with the above, we hypothesize that the foci in which the SpSHR2 receptor is found within the nucleus are regulatory sites of target genes. In addition, the accumulation of the receptor in the perinuclear region may correspond to the

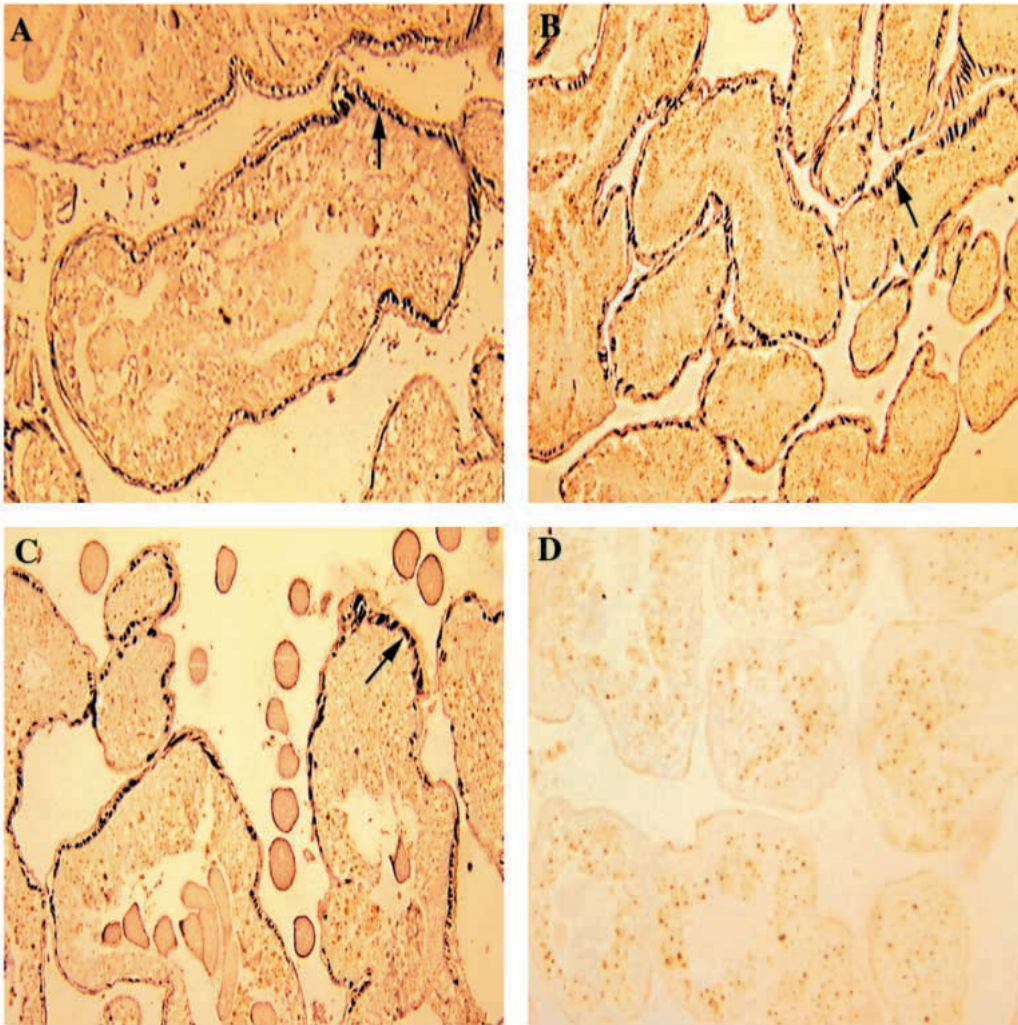


Fig. 8. Cell-type specific expression of SpSHR2 in adult tissues. Immunocytochemistry with sections of ovaries (A,C,D) and testes (B). (A,B) SpSHR2 antibody; (C) myosin antibody; (D) preimmune serum. 7 μ m sections from paraffin embedded tissue were incubated with an anti-rabbit alkaline-phosphatase conjugated secondary antibody and photographed at $\times 10$ magnification.

sites of synthesis of the receptor in the rough endoplasmic reticulum. It is also possible that the foci observed in the cytoplasm represent specific sites where the receptor is stored in an unliganded form, or where it undergoes post-translational modifications such as phosphorylation (Orti et al., 1992; Kuiper and Brinkmann, 1994).

During late embryogenesis SpSHR2 molecules reside in both the nuclear and the cytoplasmic compartment of the cells. Furthermore, as the immunoblot data indicated SpSHR2 is present in seemingly equivalent amounts throughout embryonic development. However, as shown by confocal microscopy, at the pluteus stage the number of both nuclear and cytoplasmic SpSHR2 foci is considerably smaller per individual cell, compared to the blastula and gastrula stages, in every cell type except the epithelial cells of the mouth. This apparent difference is presumably due to the dilution that occurs in the concentration of SpSHR2 molecules per cell, as the number of embryonic cells and the overall volume of the late embryo increases. The epithelial cells which constitute the pluteus mouth, however, form a thickening with a compression of their apical side. We believe this to be the reason for the apparent higher concentration of SpSHR2 in the mouth cells, compared to the rest of the oral ectoderm.

The SpSHR2 protein present in 72-hour plutei is degraded

soon after this developmental stage, since we do not detect the protein in any tissue four days later, after feeding has begun, in the 1-week-old larva. We also do not detect the protein for about another four weeks of larval development, in either tissue sections or whole mount immunofluorescence experiments. Unless the protein is in extremely low and undetectable levels or very unstable in larvae, we assume that SpSHR2 is not expressed during this 4-week long developmental period. SpSHR2 is detected again later in development (presumably following a specific gene activation event) at the 35-day old larva in a tissue specific manner. At this stage the receptor is exclusively detected at the apical side of epithelial cells in the mouth and the epaulettes of the larvae (Pearse and Cameron, 1991). The mouth epithelium and the epaulettes are highly ciliated organs. The cilia that are present in these structures are, respectively, involved in the collection and 'testing' of food and the ability of the larva to swim. These larval structures though, are formed about thirty days before any SpSHR2 protein is detected. Whether the cells expressing the SpSHR2 receptor differentiate specifically at about the 35-day-old larva or are preexisting cells which begin expressing SpSHR2 at this developmental time is not known.

The SpSHR2 receptor was also detected in different adult tissues. The exact cell type which expresses SpSHR2 in ovaries

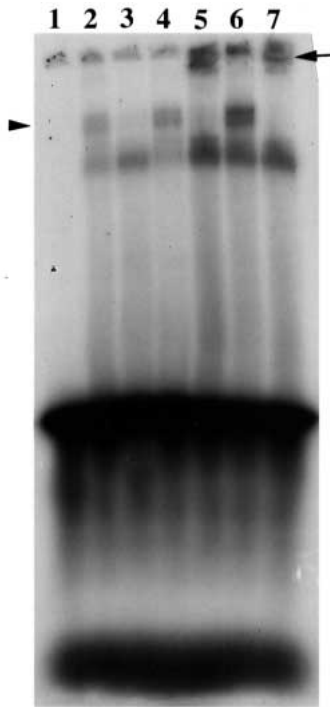


Fig. 9. SpSHR2 binds to the upstream promoter region of the CyIIIb gene. EMSA using blastula nuclear extracts (BNE) and C1R labeled oligonucleotide as probe. Lane 1: probe only; 2: BNE plus C1R probe; 3: BNE plus C1R probe plus 500-fold excess of unlabeled C1R; 4: BNE plus C1R probe plus 500-fold excess of an unrelated oligonucleotide; 5: BNE plus C1R probe plus SpSHR2 antibody; 6: BNE plus C1R probe plus preimmune serum; 7: BNE preincubated with the SpSHR2 antibody plus C1R probe. Arrowhead denotes the position of the specific SpSHR2 complex (two bands). The arrow marks the position of the supershifted complex after the addition of SpSHR2 antibodies (5, 7). Bands below the arrowhead are formed by non-specific complexes.

(Mona et al., 1991; Millonig et al., 1968) and testes (Ward and Nishioka, 1993) was discerned by immunocytochemistry of tissue sections. These results demonstrated that SpSHR2 is specifically expressed in the myoepithelial cells that surround the ovarioles and the acini. This layer of myoepithelial cells has both a sensory and contractile activity and is responsible for the spawning of the mature ova and sperm. Furthermore, it has been reported that the myoepithelial cells of echinoderms, as well as of other animals, may carry a rudimentary cilium that has a sensory function (Gardiner and Rieger, 1980). These ciliated cells, as well as the ones that form the mouth and epaulettes, may be capable of conducting stimuli to the interior of the cell and subsequently to other tissues of the embryo and adult animal. Thus, in the late embryo, larva and adult animal, the function of SpSHR2 may necessitate its specific localization apically in ciliated epithelial cells of different tissues or organs, which have a sensory and/or contractile activity. It is possible that there is a domain of the receptor which is responsible for this cytoplasmic apical localization, or that SpSHR2 has an affinity to other proteins also positioned at the apical side of epithelial cells.

In the early embryo, the maternal SpSHR2 receptor, which is present in all nuclei, may function as a transcription factor,

which alone or in co-operation with a number of other cell-type specific transcription factors regulates, either by activation or repression, the expression pattern of target genes such as the CyIIIb actin (see also Xu et al., 1996). As shown in the present study, SpSHR2 receptor present in blastula nuclear extracts binds in vitro the C1R element located in the upstream promoter region of the CyIIIb actin gene. This element is necessary for suppression of the CyIIIb actin gene in mesodermal and endodermal cells, restricting its expression in the ectoderm. The cooperation of two additional elements (C1L and E1) with C1R, is required for the confinement and enhancement of the CyIIIb expression in the cells of the aboral ectoderm at the pluteus stage (Xu et al., 1996). The two bands that were observed in the EMSA experiment may represent complexes formed by different SpSHR2 isoforms, or by SpSHR2 and another interacting protein. As previously shown (Kontrogianni-Konstantopoulos et al., 1996), alternative splicing of the SpSHR2 primary transcript results in the generation of different isoforms of the receptor, which contain the DNA binding domain. If the CyIIIb gene is an in vivo target of the SpSHR2 receptor, we can speculate that the different SpSHR2 isoforms may have distinct effects on its expression in the early embryo.

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